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Immunochemical Identification and Preliminary Characterization of a Nonfimbrial Hemagglutinating Adhesin of *Bacteroides gingivalis*

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A cell-bound hemagglutinating adhesin (HA-Ag2) of *Bacteroides gingivalis* was identified by crossed immunoaffinity electrophoresis as one of the common antigens of the species. A polyclonal antiserum with a restricted specificity for HA-Ag2 was produced by immunizing with the relevant immunoprecipitate excised from crossed-immunoelectrophoresis gels. The immunoglobulin G fraction of this monospecific antiserum inhibited hemagglutination. The antiserum was used against a cell surface extract of *B. gingivalis* in immunoblotting experiments, and we detected two antigens with apparent molecular masses of 33 and 38 kilodaltons in *B. gingivalis* ATCC 33277 and W83. Monoclonal antibody C1.17, produced in another laboratory against *B. gingivalis* 381 and characterized as showing reactivity with a hemagglutinin of this strain (Y. Naito, K. Okuda, T. Kato, and I. Takazoe, Infect. Immun. 50:231–235, 1985), was also used to produce immunoblots of extracts of strains ATCC 33277 and W83. The apparent molecular masses of the major polypeptides recognized by monoclonal C1.17 in the immunoblots were the same as those detected by the polyclonal monospecific antiserum, i.e., 33 and 38 kilodaltons. Significantly, none of the polypeptides identified in this study corresponded to the polypeptide appearing in the 41- to 43-kilodalton region and identified by Yoshimura and co-workers (F. Yoshimura, K. Takahashi, N. Yoshinobu, and T. Suzuki, J. Bacteriol. 160:949–957, 1984) as the fimbrial protein characteristic of the species. Enzyme-linked immunosorbent assay inhibition experiments with the monospecific antiserum indicated that the cell surface extracts from strains ATCC 33277 and W83 were strong inhibitors, whereas the fimbria-enriched preparations from both strains failed to inhibit binding of antibodies to the cell surface antigens. As a whole, our study indicates that a nonfimbrial surface protein complex demonstrating erythrocyte-binding capacity, HA-Ag2, is common to three strains of *B. gingivalis* and is composed of at least two associated polypeptides with apparent molecular masses of 33 and 38 kilodaltons which share at least one antigenic determinant.

Hemagglutination is a distinctive characteristic of *Bacteroides gingivalis* which allows its differentiation from other asaccharolytic, black-pigmented *Bacteroides* species (22). Erythrocytes of numerous animal species (including human erythrocytes, regardless of their ABO blood type) are agglutinated by *B. gingivalis* (18). This hemagglutination is not inhibited by mannose or by a dozen sugars and oligosaccharides (18), suggesting that it is not a lectinlike activity. Data from Inoshita et al. (9) on an exohemagglutinin found in the culture medium of *B. gingivalis* 381 and from Okuda et al. (19) on a hemagglutinin prepared from the supernatant of broth culture from the same strain do not agree on the protein nature of the hemagglutinins studied. Thus it may very well be, as suggested by Boyd and McBride (4), that the hemagglutinin of *B. gingivalis* is a lipid-protein complex.

It is generally accepted that bacteria that cause hemagglutination possess adhesins on their cell surface and that the presence of bacterial surface adhesins is often essential to the pathogenesis of many infectious diseases. Since the pathogenic potential of *B. gingivalis* seems to stem from adhesive interaction with eucaryotic or procaryotic cell surfaces (23, 24), such interactions, possibly mediated by adhesins, are of particular interest. Although data suggest that fimbriae of *B. gingivalis* mediate hemagglutination (18, 24), there is conflicting evidence (4, 19, 26) that a nonfimbrial hemagglutinin may be present on the cell surface of the bacterium. Inoshita et al. (9) and Okuda et al. (19) have reported on the isolation of hemagglutinins from culture supernatants of *B. gingivalis* 381 which may not be identical, suggesting that the species may possess more than one hemagglutinin. Furthermore, although the importance of other hemagglutinins of gram-negative bacteria in pathogenicity is well documented, the relative importance, if any, of *B. gingivalis* hemagglutinin(s) in periodontal pathogenesis still remains to be assessed. For such studies, a preliminary characterization of the hemagglutinin is required.

The objective of this study was to identify and immunochemically characterize the bacterial surface component(s) responsible for adherence to erythrocytes, the first step in cell association leading to hemagglutination. The modification of crossed immunoelectrophoresis for ligand-receptor characterization described by Bjerrum and co-workers (2), and known as crossed immunoaffinity electrophoresis (CIAE), enabled the identification of one hemagglutinating adhesin (HA-Ag2) among the mosaic of antigens on the cell surface of *B. gingivalis*. Preliminary characterization of HA-Ag2 involved other immunochemical procedures, including immunoblotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of a cell surface extract, with a monospecific antiserum produced against this salient antigen.

In this communication the term receptor will be used in a rather loose way and should be understood as equivalent to binding site, since in our effort to identify the bacterial recognition molecules responsible for bacteria-erythrocyte interaction, no attempt has been made to distinguish between ligand and receptor according to classical criteria for such interactions (7). Hemagglutinating adhesin refers to an

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antigen present on the cell surface of *B. gingivalis*, whereas receptor refers to the complementary structure in erythrocyte membranes, which is recognized and bound by the hemagglutinating adhesin.

MATERIALS AND METHODS

Bacteria and growth conditions. *B. gingivalis* ATCC 33277 and W83 and *Bacteroides asaccharolyticus* ATCC 25260 were from our stock collection. The strains were anaerobically maintained on blood agar plates (13) enriched with hemin (10 μg/ml) and vitamin K_1 (5 μg/ml) and grown in enriched Trypticase (BBL Microbiology Systems)-yeast extract broth. They were checked for purity by Gram reaction, inoculation of a duplicate set of blood agar plates for aerobic and anaerobic incubation, and by the API-Zym enzymatic tests (Analytab Products) (12, 21).

Bacterial extracts. An EDTA cell surface extract was obtained from washed cells by application of mild attrition forces with glass beads (glass bead-EDTA extract [GBE]) as previously described (20). A fimbria-enriched fraction was obtained by the technique of Yoshimura et al. (26).

Production of antiserum. Antisera against the cell surface antigens (GBE) were produced as previously described (20). A monospecific antiserum was produced by injecting a rabbit with a selected immunoprecipitate obtained by crossed immunoelectrophoresis (CIE). Briefly, the selected immunoprecipitate was excised from seven thoroughly pressed and washed replicate CIE gels, minced, and placed in 2.5 ml of 0.01 M phosphate-buffered saline (PBS), pH 7.6. The suspension was sonicated, mixed with 2.5 ml of Freund incomplete adjuvant, and injected into one rabbit, 1 ml intramuscularly in each hind limb and 3 ml subcutaneously at several sites on the back. After 2 weeks, the selected immunoprecipitate from four gels was mixed with 1 ml of PBS and 1 ml of Freund incomplete adjuvant and injected intradermally at several sites on the neck and back. A similar booster was given after 2 weeks, and the animal was bled 1 week later. Young (less than 2 kg in weight) female rabbits, whose preimmune serum showed no reactivity with GBE by immunoblotting, were selected for this purpose. Antisera against whole cells of *Lactobacillus casei* L324M and *Bacterionema matruchotii* ATCC 14266 were from our stock collection. Gamma globulin fractions were purified from serum by three precipitations with 70% saturated ammonium sulfate, and the immunoglobulin G (IgG) fractions were obtained by additional chromatography on DEAE-cellulose.

Solubilization of erythrocyte membranes. Washed human erythrocytes were lysed in 10 mM Tris–1 mM EDTA (pH 8.0) by gentle stirring for 5 min at room temperature. The membranes were sedimented at 12,000 × *g* for 30 min and washed at 4°C in this buffer until free of hemoglobin. For membrane solubilization, the erythrocyte ghosts were suspended in 5 mM phosphate buffer (pH 8.0) at a protein concentration of 5 mg/ml, and Triton X-100 was added to a final concentration of 2% (vol/vol). The suspension was vortexed at room temperature for 5 min and then centrifuged at 40,000 × *g* for 5 h. The supernatant was designated Triton X-100-solubilized erythrocyte membranes (TSEM).

Immunoelectrophoretic methods. CIE was performed as described previously (20) with a blank intermediate gel on all plates to increase resolution. Modifications of CIE to study ligand-receptor interactions were introduced in the first-dimension electrophoresis as described by Bøg-Hansen et al. (3) and Bjerrum et al. (2). This methodology is known as CIAE. Solubilized erythrocyte membranes at various concentrations were incorporated into the first-dimension gel in which the bacterial extract was to be electrophoresed.

SDS-PAGE. SDS-PAGE was carried out on 0.75-mm-thick slab gels in the buffer system of Laemmli (11) at 15 mA per gel with a 12% (wt/wt) polyacrylamide separating gel overlaid with a 5% stacking gel. Samples were boiled for 4 min in dissociating buffer containing 2% SDS and 5% β-mercaptoethanol. After electrophoresis, the polypeptides were stained by the potassium permanganate-silver nitrate procedure of Ansorge (1).

Immunoblot analysis. The separated proteins were blotted onto nitrocellulose paper by electrophoretic transfer at 0.1 A overnight as described by Towbin et al. (25), in 25 mM Tris–192 mM glycine–20% (vol/vol) methanol (pH 8.3). To monitor the efficiency of the protein transfer, a reference nitrocellulose strip containing molecular weight standards and bacterial extracts was stained with amido black. For immunological detection on blot transfers, all steps were carried out at room temperature in Tris-buffered saline (TBS) with gentle orbital agitation. The blotted nitrocellulose strips were quenched for 60 min in TBS containing 3% gelatin and then incubated for 2 h with antiserum or ascites fluid diluted in 1% gelatin–TBS. After strips were washed twice for 15 min in TBS containing 0.05% Tween 20, they were incubated for 1 h with an IgG fraction of peroxidase-conjugated antibody to rabbit IgG or to mouse IgM diluted 1:2,000 in 1% gelatin–TBS. The strips were washed twice for 15 min, and the peroxidase reaction was initiated by placing the strips in a methanol solution containing the chromogenic substrate 4-chloro-1-naphthol (0.5 mg/ml) and 0.02% (vol/vol) H_2O_2. The enzymatic reaction was stopped by washing the strips in distilled water.

Polypeptide analysis by SDS-PAGE of individual immunoprecipitates excised from CIE gels was performed by the method of Norrild et al. (17), followed by immunoblotting. Briefly, 60 μl of GBE from strain ATCC 33277 was reacted with homologous antiserum (6.25 μl/cm^2) in CIE. The gels were pressed and washed twice in 0.15 M NaCl and once in distilled water. The precipitates were visualized by staining overnight in a 0.01% aqueous solution of Coomassie brilliant blue and destaining in water. Immunoprecipitate 2 from two replicate gels was excised and collected in a centrifuge tube. For elution, 150 μl of sample buffer (11) was added, and the agarose was homogenized by sonication for 30 s (microtip, energy level 5, 50% duty cycle; Sonifier cell disruptor W-350; Heat Systems Ultrasonics Inc.). The gel homogenate was extracted by gentle shaking for 2 h at 37°C, and the agarose was pelleted by centrifugation at 12,500 × *g* for 5 min. The supernatant was used for SDS-PAGE and subsequent immunoblot analysis.

ELISA and ELISA inhibition. Enzyme-linked immunosorbent assays (ELISAs) were performed with 96-well microtiter trays as described previously (14). Briefly, each well was coated with 100 μl of GBE antigen from strain ATCC 33277, diluted to give a protein concentration of 5 μg/ml. The immune reaction was performed in duplicate wells by adding 100 μl of serially diluted monospecific rabbit antiserum. Heavy-chain-specific goat IgG anti-rabbit immunoglobulin conjugated to alkaline phosphatase (IgG specific; Jackson Laboratories) was diluted 1:3,000 and incubated in the wells for 75 min at 37°C. Reactivity was revealed by adding the substrate *p*-nitrophenylphosphate (type 104; Sigma) and reading at 410 nm (MicroElisa minireader; Dynatech Laboratories, Inc.). Inhibition of the monospecific antiserum reacting with hemagglutinin fragments was performed by incubation of a 1:100 dilution of the antiserum with the

inhibitors. Each inhibitor was mixed with the antiserum to obtain a serial dilution ranging from 2.94 to 180 μg/ml, and the mixtures were incubated for 30 min at room temperature. After centrifugation at 15,000 × *g* for 2 min, the antibody not neutralized by the inhibitors was measured by ELISA. The 50% inhibitory value was recorded as the concentration of inhibitor needed to obtain a 50% decrease in the optical density as compared with that in controls with no inhibitor added.

Colloidal gold marker. Colloidal gold (apparent size of particles, 15 to 20 nm) was prepared by reduction of chloroauric acid with sodium citrate by a procedure similar to that of Horisberger (8). To 100 ml of the gold particles was added 135 μl of the TSEM preparation at a protein concentration of 1 mg/ml, and the mixture was incubated for 2 h at room temperature. Then 60 μl of 1% polyethylene glycol (3,350 molecular weight) was added, the mixture was centrifuged in the cold at 12,000 × *g* for 45 min, and the pellet was suspended in 15 ml of PBS containing 0.02% (vol/vol) polyethylene glycol. The gold particles stabilized with the solubilized erythrocyte membranes (gold-TSEM) were kept at 4°C.

Electron microscopy. For topographical analysis of the adhesin-receptor interaction, 1 drop of bacterial cell suspension in PBS (about 1.2×10^9 cells per ml) was deposited on a Formvar-coated, carbon-reinforced copper grid and incubated with 10 μl of gold-TSEM marker for 30 min at room temperature. Three negative controls were used. Control grids of bacterial suspension received 1 drop of PBS containing 0.02% polyethylene glycol. Other grids were incubated with a polyethylene glycol-gold complex. Finally, a third set of grids was preincubated with unconjugated TSEM before incubation with the gold-TSEM marker. The grids were blotted dry and incubated with 2% phosphotungstic acid in 0.15% (wt/vol) bovine serum albumin (pH 7.2) in deionized water. Excess liquid was removed with filter paper, and the grids were air dried before examination. Negative staining of preparations with phosphotungstic acid was performed as previously described (20). Specimens were examined with a Philips EM 300 transmission electron microscope operating at 60 kV.

Hemagglutination and hemagglutination inhibition assays. Samples (50 μl) of a 1.5% suspension of formalinized human erythrocytes in PBS containing 0.05% (wt/vol) sodium thioglycolate were added to an equal volume of bacterial suspension diluted serially twofold in the same buffer (starting with 2×10^9 cells per ml). After gentle mixing, the microdilution plate was incubated at room temperature for 60 min. The hemagglutination titer was estimated as the reciprocal of the highest dilution showing a positive reaction. Inhibition by antisera or solubilized erythrocyte membranes was tested after preincubation of bacteria or bacterial extract with appropriately diluted antiserum, or TSEM diluted 1:500, for 120 min at 37°C.

Modification of bacterial extracts. The sensitivity of the GBE extract to proteolysis was determined by incubation with alkaline protease and proteinase K. Protease from *Streptomyces griseus* (type XXI; Sigma) was dissolved at a concentration of 200 μg/ml in 50 mM Tris (pH 10.4), and 200 μl was added to 200 μl of bacterial extract. This mixture was incubated for 60 min at 30°C. Proteinase K (type XI; Sigma) was dissolved in distilled water at a concentration of 1 mg/ml, 200 μl was added to 200 μl of bacterial extract, and the mixture was incubated for 120 min at 37°C. The enzymes were inactivated by addition of phenylmethylsulfonyl fluoride at a final concentration of 1 mM. Controls received buffer alone or distilled water alone, as appropriate. A solution of 0.5 M sodium metaperiodate (Sigma) was prepared in distilled water, and 200 μl was added to 200 μl of bacterial extract. Controls received 200 μl of distilled water. The oxidation was carried out at 4°C in the dark. Heat sensitivity was tested by incubating the bacterial extract at 100°C in a water bath for 10 min. The pretreated samples were then tested for hemagglutinating activity in parallel with untreated samples.

Immunofluorescence. To 50-μl samples of a 1.5% suspension of formalinized human erythrocytes in PBS was added 50 μl of a serial twofold dilution of GBE from strain ATCC 33277. After gentle mixing, the suspension was incubated at room temperature for 60 min. From each suspension, 10 μl was spread on a glass slide, dried, and heat fixed. Each smear was then incubated with either antiserum 8503 or the reference *B. gingivalis* antiserum and further processed for indirect immunofluorescence as previously described (15). Whole cells of *B. gingivalis* ATCC 33277 were used as positive controls. Negative controls included erythrocytes incubated with GBE extracted from *B. asaccharolyticus* ATCC 25260 and the use of preimmune serum or antisera against whole cells of *L. casei* and *B. matruchotii*.

Protein determination. The amount of protein in the various extracts was routinely determined using the protein assay kit from Bio-Rad Laboratories based on the protein assay of Bradford (5). Bovine serum albumin (fraction V) was used as the reference standard.

RESULTS

Bacterial and erythrocyte extracts are functional. The hemagglutination titer of the whole-cell suspension (0.2 g/ml) was 1,280, and heating the cells at 100°C for 10 min totally abolished the hemagglutinating activity. The average hemagglutination titer of the GBE extracts from both strains ATCC 33277 and W83 (0.8 mg/ml) was 640; it was reduced to a titer of 40 by heating at 37°C and was entirely abrogated at 100°C. Protease- and proteinase K-treated extracts exhibited no hemagglutinating activity. In contrast, treatment with sodium metaperiodate did not modify the activity. These results suggest that the hemagglutinin of *B. gingivalis* contained in the EDTA cell surface extract is a proteinaceous substance.

Several lines of evidence indicated that the TSEM preparation contained, in an active form, the receptor bound by *B. gingivalis* cells. First, hemagglutination inhibition assays indicated that the TSEM preparation reduced to an endpoint titer of 1:40 the hemagglutinating activity of a bacterial suspension that was positive at a titer of 1:640. The same preparation diluted 1:160 induced a total inhibition of the hemagglutinating activity of the GBE, which normally agglutinated erythrocytes with an endpoint titer of 1:128. Second, gold-labeled TSEM incubated with *B. gingivalis* cells demonstrated numerous adhesin-receptor interactions at the cell surface of the bacteria (Fig. 1A). These positive reactions were inhibited by a preincubation of the bacteria with the unconjugated TSEM before incubation with the gold-TSEM probe (Fig. 1B). The gold-TSEM probe incubated with cells of *B. asaccharolyticus*, a nonhemagglutinating species, did not show binding to the latter (Fig. 1C). The ease with which the gold-TSEM probe could be adsorbed to *B. gingivalis* cells and the density of the labeling suggest a cell surface location for the hemagglutinin. Noteworthy was the observation that no fibrillar material reminiscent of fimbriae appeared to be labeled.

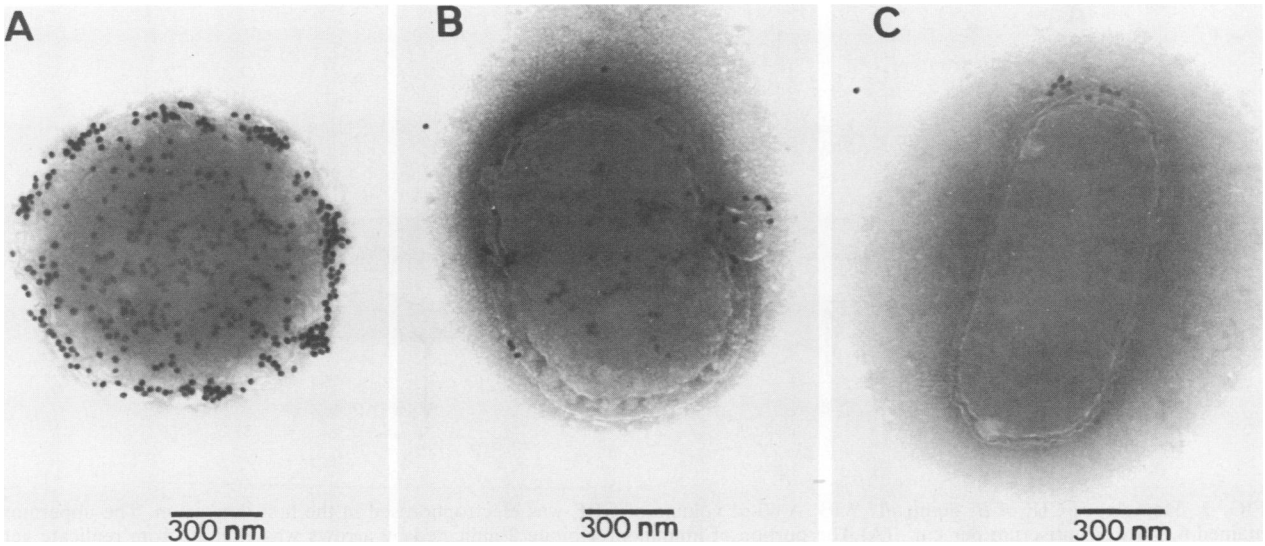

FIG. 1. Arrangement of the adhesin specific for erythrocytes on the surface of *B. gingivalis* as determined by gold labeling. Colloidal gold (15- to 20-nm apparent particle size) was stabilized with TSEM, and the resulting probe was incubated with bacteria for transmission electron microscopy. (A) Intense positive labeling of *B. gingivalis* ATCC 33277 demonstrated numerous adhesin-receptor interactions scattered on the outer surface of the cell. (B) Preincubation of *B. gingivalis* ATCC 33277 with unconjugated TSEM before incubation with gold-TSEM inhibited binding of the probe. (C) *B. asaccharolyticus* ATCC 25260, a nonhemagglutinating species, incubated with gold-TSEM was not labeled. Binding of the probe to cells of *B. gingivalis* only demonstrates and localizes the hemagglutinating adhesin at the outer surface of the bacteria. No fibrillar material reminiscent of fimbriae appeared to be labeled.

CIAE. CIE patterns developed as controls in our experiments revealed at least 20 discrete immunoprecipitates, identical to those of the reference pattern established for *B. gingivalis* in a previous study (20). Deviations from this pattern upon introduction of solubilized erythrocyte membranes into the system were interpreted as evidence of ligand-receptor interaction. A distinct change in the migration velocity of one immunoprecipitate was observed when the bacterial extract was electrophoresed through a first-dimension gel containing a uniform concentration of solubilized erythrocyte membranes (Fig. 2). The retardation of the anodal migration of immunoprecipitate 2 (numbered in accordance with the established reference pattern of Parent et al. [20]) was increased with increasing concentrations of TSEM. This positive CIAE reaction thus revealed an affinity between a surface antigen of *B. gingivalis* and a complementary binding site present in the solubilized erythrocyte membranes. We infer from this biospecific interaction that anti-

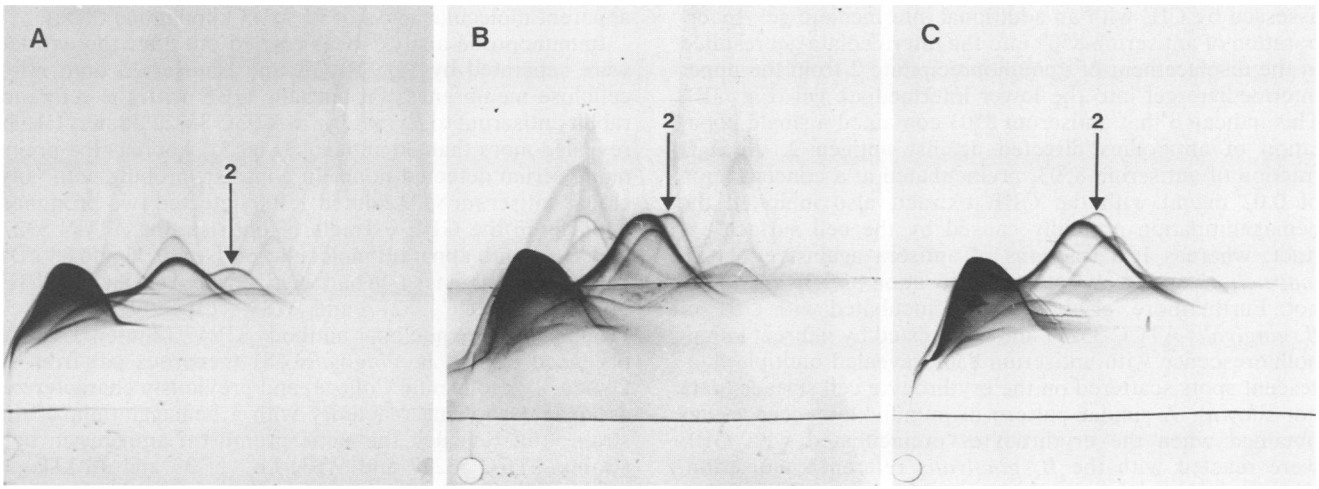

FIG. 2. CIAE of GBE from *B. gingivalis* ATCC 33277. GBE (60 μl) was electrophoresed through the first-dimension gel containing TSEM. Antiserum to *B. gingivalis* ATCC 33277 (6.25 μl/cm^2) was incorporated into the second-dimension gel. (A) Control, no TSEM was incorporated into the first dimension gel; the CIE pattern obtained was similar to the reference pattern previously established for *B. gingivalis*. (B) TSEM (6.25 μl/cm^2) was incorporated into the first-dimension gel. (C) TSEM (25 μl/cm^2) was incorporated into the first-dimension gel. A progressive retardation of the anodal migration of one immunoprecipitate (arrow), identified as immunoprecipitate 2 of the previously established reference pattern for *B. gingivalis*, was observed with increasing concentrations of TSEM. The ligand-receptor interaction thus evidenced suggests that antigen 2 of *B. gingivalis* is endowed with an erythrocyte-binding capacity.

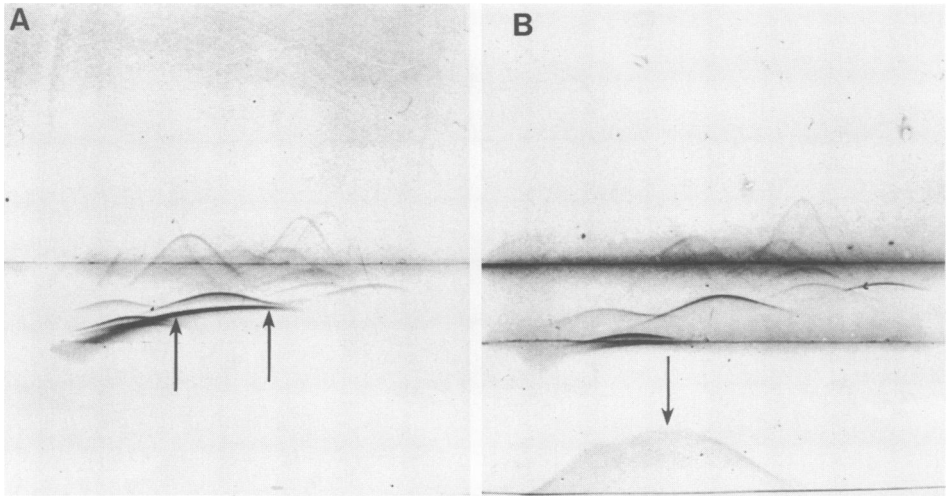

FIG. 3. Homolgous CIE of *B. gingivalis* W83. A 60-μl volume of GBE was electrophoresed in the first dimension. The uppermost gel contained 6.25 μl of antiserum per cm^2. (A) The portion of immunoprecipitate 2 indicated by arrows was excised from replicate gels for immunization to produce antiserum 8503; a blank intermediate gel was inserted to counteract electroendosmosis flow resulting in undesired migration of antibodies and to improve resolution; a second antibody-free intermediate gel was inserted as a control for panel B. (B) CIE with an intermediate gel containing 31.25 μl of antiserum 8503 per cm^2; disappearance of immunoprecipitate 2 from the reference pattern and appearance of a single immunoprecipitate (arrow) in the lower, antibody-containing intermediate gel indicates that antiserum 8503 contains a single population of antibodies against antigen 2.

gen 2 of the reference CIE pattern is identical to or closely associated with a hemagglutinating adhesin of *B. gingivalis* (HA-Ag2).

Production of monospecific antiserum 8503. Our approach to the study of HA-Ag2 was to generate an antiserum with a specificity restricted to this antigen. This was obtained by excision of immunoprecipitate 2 from CIE gels for immunization. Antigen 2 was best resolved with GBE of strain W83 as the test antigen, reacted with homologous antiserum (Fig. 3A). Only the major portion free of obvious intersecting immunoprecipitates was used for immunization. A short immunization procedure of 5 weeks' duration was used to ensure specificity. The specificity of the antiserum thus obtained, henceforth referred to as antiserum 8503, was assessed by CIE with an additional intermediate gel. Incorporation of antiserum 8503 into the intermediate gel resulted in the displacement of immunoprecipitate 2 from the upper intermediate gel into the lower intermediate gel (Fig. 3B). This indicated that antiserum 8503 contained a single population of antibodies directed against antigen 2. An IgG fraction of antiserum 8503, preincubated at a concentration of 0.02 mg/ml with the GBE extract, also inhibited the hemagglutination normally caused by the cell surface extract, whereas IgG fractions of antisera against either *B. matruchotii* or *L. casei* at a concentration of 0.35 mg/ml did not. Furthermore, erythrocytes preincubated with GBE of *B. gingivalis* ATCC 33277 and then tested by indirect immunofluorescence with antiserum 8503 revealed multiple fluorescent spots scattered on the erythrocyte cell surface (data not shown). A similar pattern of immunofluorescence was obtained when the erythrocytes preincubated with GBE were reacted with the *B. gingivalis* reference antiserum, whereas preimmune or irrelevant antisera showed a negative reaction. The above experiments showed (i) that a subfraction of the GBE extract contained adhesin fragments binding to erythrocytes, (ii) that the erythrocyte-bound bacterial components reacted with antiserum 8503, and (iii) that the erythrocyte-binding adhesins of *B. gingivalis* W83 and ATCC 33277 were antigenically related. These observations indicate that antiserum 8503 contained predominantly antibodies with a specificity for the hemagglutinin associated with antigen 2 of *B. gingivalis* (HA-Ag2).

SDS-PAGE and immunoblot analysis. The presence of fibrillar material reminiscent of fimbriae has already been observed in GBE extract (20). Similarly, negative staining of the fimbria-enriched fraction prepared by the method of Yoshimura et al. (26) revealed a dense network of fimbrialike structures (Fig. 4B). Comparative analysis of the polypeptide profiles of the GBE extract and fimbria-enriched fraction prepared from strain ATCC 33277 (Fig. 4A) revealed that both preparations contained the fimbrilin monomer typical of *B. gingivalis* reported by Yoshimura et al. (26). The fimbrilin monomer appeared as a thick, densely stained band with an apparent molecular mass of 41 to 43 kilodaltons (kDa).

Immunoprobe analysis was carried out after the extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Probing the GBE with the reference rabbit antiserum to *B. gingivalis* ATCC 33277 diluted 1:1,000 revealed more than 30 antigens (Fig. 5), whereas the preimmune serum detected none. In contrast, probing with polyclonal antiserum 8503 diluted 1:300 detected two prominent antigens in the GBE extracts of both strains ATCC 33277 and W83, with apparent molecular masses of 33 and 38 kDa. Additional antigens at 48 and 36 kDa were revealed in GBEs of strains ATCC 33277 and W83, respectively (Fig. 5). Probing with monoclonal antibody C1.17 (diluted 1:1,000), produced against *B. gingivalis* 381 (generous gift from K. Okuda, Tokyo Dental College) and previously characterized as an IgM showing reactivity with a hemagglutinin of this strain (16), revealed the same prominent antigens in both strains ATCC 33277 and W83, i.e., at 33 and 38 kDa. In contrast, the fimbria-enriched fraction extracted from strains ATCC 33277 and W83 probed with either polyclonal antiserum 8503 or monoclonal antibody C1.17 remained negative. Incubation of blots with irrelevant antisera revealed no reaction, indicating that the positive reactions described above could not be attributed to Fc receptors present in the *B. gingivalis* extract. Omission of β-mercaptoethanol from

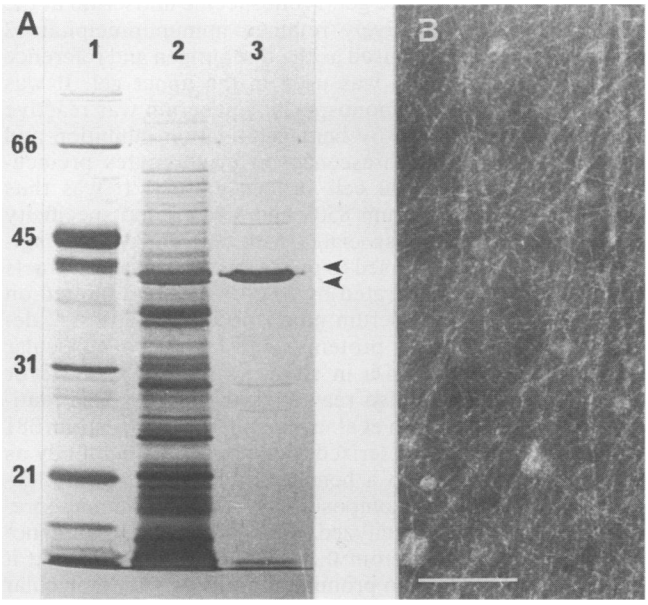

FIG. 4. (A) SDS-PAGE analysis of preparations from *B. gingivalis* ATCC 33277 on 12% polyacrylamide gels (silver stain). Lanes: 1, molecular weight standards; 2, GBE extract (15 μg of protein loaded); 3, fimbria-enriched fraction (5 μg of protein loaded). The fimbrilin monomer, characterized by an apparent molecular mass of 41 to 43 kDa and present in both preparations, is indicated by arrows. (B) Electron micrograph of phosphotungstic acid negatively stained fimbria-enriched fraction from *B. gingivalis* ATCC 33277 (bar, 100 nm).

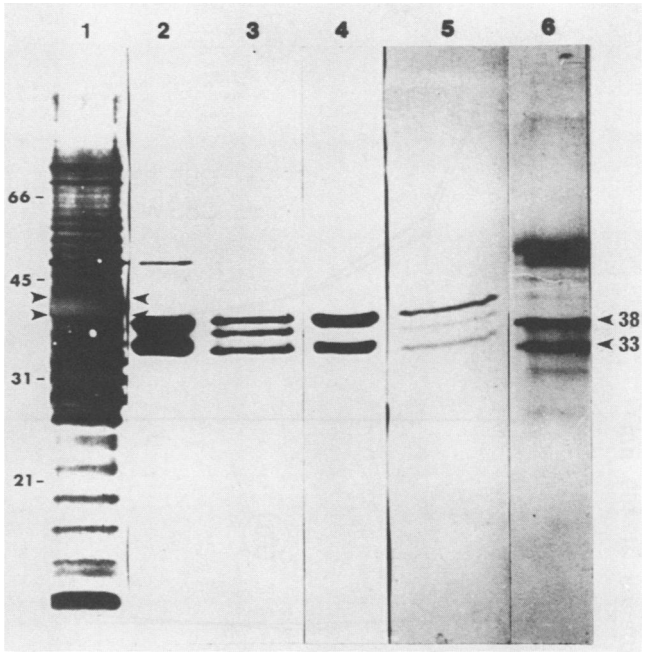

FIG. 5. Immunoblot analysis showing reactions of the GBE extracts from *B. gingivalis* ATCC 33277 (lanes 1, 2, and 4) and W83 (lanes 3 and 5) with the reference antiserum to *B. gingivalis* (lane 1), monospecific polyclonal antiserum 8503 (lanes 2 and 3), and monoclonal antibody C1.17 (lanes 4 and 5). The position of the molecular mass markers is indicated on the left. Arrows in lane 1 indicate the 41- to 43-kDa region typical of the fimbrilin of *B. gingivalis*, a prominent antigen among 30 others detected by the reference serum. Note that monospecific antiserum 8503 and monoclonal antibody C1.17 do not detect this antigen in the GBE with which they are reacted. Lane 6 reveals the polypeptide composition of antigen 2 of strain ATCC 33277 immunoprecipitated in CIE gel by reference antiserum, excised and analyzed by SDS-PAGE, and immunoblotted with monospecific antiserum 8503. The apparent molecular masses of the two major antigenic polypeptides comprising HA-Ag2 are indicated on the right.

the buffer used to solubilize samples for SDS-PAGE did not change the pattern obtained when the subsequent blots were probed with antiserum 8503, indicating that the two antigens are not likely to be linked by disulfide bonds (results not shown).

The polypeptide composition of the antigen contained in immunoprecipitate 2 excised from a CIE gel (GBE of strain ATCC 33277 reacted with homologous antiserum) was analyzed by SDS-PAGE and immunoblotting with antiserum 8503 (Fig. 5). It was composed of three different polypeptides with molecular masses of 48, 38, and 33 kDa. A major polypeptide was observed at 50 kDa and corresponded to the heavy-chain subunit of the IgG molecules present in the immunoprecipitate.

Results from the immunoblotting experiments indicated the following: (i) a major population of antibodies in the polyclonal antiserum 8503, raised against an immunoprecipitated antigen showing erythrocyte-binding capacity, had the same specificity as monoclonal antibody C1.17, and both identified HA-Ag2 of *B. gingivalis*; (ii) these antibodies had an affinity for the same antigen (or epitope) present on several polypeptides that differ in molecular mass; (iii) antigenic cross-reactivity of the polypeptides was high within three strains of *B. gingivalis*, namely, ATCC 33277, W83, and 381; and (iv) these antibodies did not react with components of fimbria-enriched fractions from strains ATCC 33277 or W83. By taking advantage of the specificity of these immunoreagents, we were thus able to demonstrate that HA-Ag2 may be made up of a protein complex composed of at least two associated polypeptides that are not linked by disulfide bonds. The two polypeptides were antigenically similar and shared at least one epitope.

ELISA inhibition. ELISA inhibition of monospecific antiserum 8503 was performed with GBE and fimbriae, prepared from *B. gingivalis* ATCC 33277 and W83, as inhibitors. GBE from strains ATCC 33277 and W83 strongly inhibited antiserum 8503 (Fig. 6); the amounts of inhibitor needed to obtain 50% inhibition of the antibodies were 65 and 55 μg/ml, respectively. In contrast, the fimbria-enriched preparation from both strains totally failed to inhibit binding of antibodies of antiserum 8503 to the cell surface antigens coating the microdilution plate. These results indicate that monospecific antiserum 8503 does not contain antibodies that react with the fimbriae of *B. gingivalis*.

DISCUSSION

Most methods used for studies of bacterial adhesins are preparative in nature; the ligand must be purified before its characterization. In this study, we employed CIE and its modification, CIAE, used for ligand-receptor characterization by Bjerrum and co-workers (2), as the analytical reference methods. These are nondenaturing techniques, allowing protein antigens to remain in their native state and to retain their biological activity so that biospecific interactions, in this case ligand-receptor interactions, are not impaired. We were successful in identifying antigen 2 of the reference CIE pattern of *B. gingivalis* (20) as one hemagglutinating adhesin among the mosaic of antigens on the cell

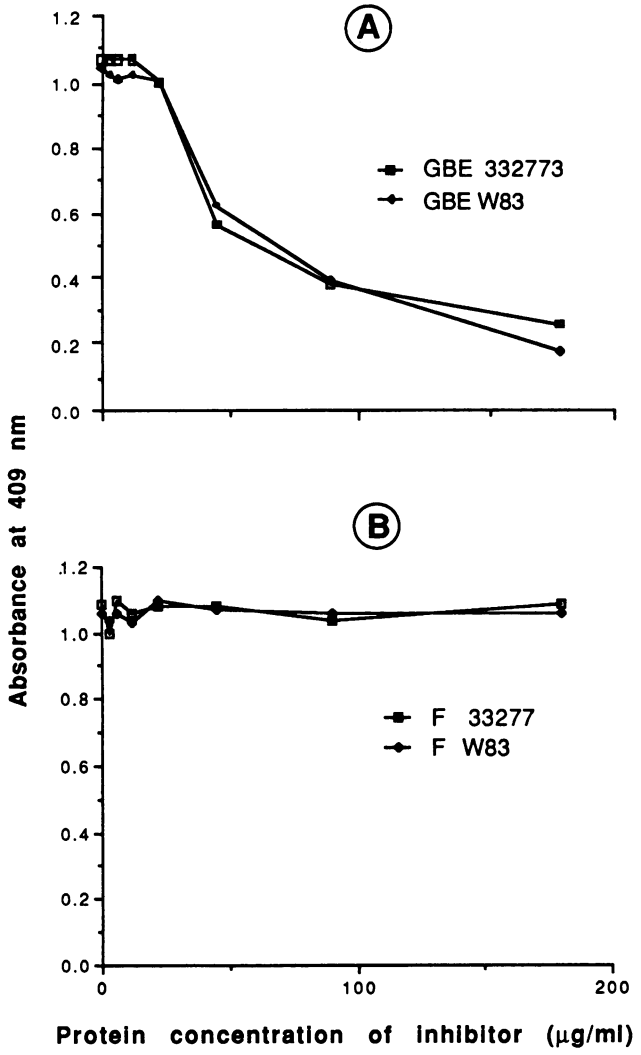

FIG. 6. ELISA inhibition of monospecific antiserum 8503, produced against the hemagglutinin associated with antigen 2 of *B. gingivalis*, with GBE (A) or fimbriae (B) from strain ATCC 33277 (□) or W83 (●). The microdilution tray was coated with 5 μg of GBE from strain ATCC 33277 per ml.

surface of *B. gingivalis*. This adhesin was termed HA-Ag2. This functional identification was made possible by recognizing the selective capacity of HA-Ag2 to form an affinity precipitate with solubilized erythrocyte membranes during electrophoresis. To obtain this result, it was first necessary to obtain two lines of evidence. Evidence was first obtained indicating that the bacterial cell surface preparation extracted with EDTA contained the hemagglutinin, as demonstrated by hemagglutination assays and by positive immunofluorescence of erythrocytes incubated with GBE. Evidence was also obtained that the receptor on erythrocytes was present in the solubilized erythrocyte membranes by hemagglutination inhibition assays and by the demonstration in the electron microscope of gold-receptor particles adsorbing onto bacterial cells.

An important step in our immunochemical characterization of HA-Ag2 was the production of a monospecific antiserum by immunization with immunoprecipitate 2 excised from CIE gels. Evidence that the antiserum thus produced was monospecific for antigen 2 was obtained by CIE with an intermediate gel. Monospecific antiserum in the intermediate gel selectively retained immunoprecipitate 2 when GBE extract was used as the test antigen and reference *B. gingivalis* antiserum was used in the upper gel. It was further shown that the monospecific antiserum was reactive with the hemagglutinin by hemagglutination inhibition and by positive immunofluorescence on erythrocytes preincubated with the bacterial cell surface extract. It was thus established that antiserum 8503 had a restricted specificity for the hemagglutinin associated with antigen 2 of *B. gingivalis* (HA-Ag2). When used to probe the cell surface extracts from *B. gingivalis* separated by SDS-PAGE and blotted on nitrocellulose, the antiserum monospecific for HA-Ag2 detected two prominent proteins with apparent molecular masses of 33 and 38 kDa in either strain ATCC 33277 or W83. These antigens also reacted with a monoclonal antibody produced by Naito et al. against *B. gingivalis* strain 381 (16). Naito et al. characterized this monoclonal antibody as showing reactivity with a hemagglutinin of this strain. Finally, the polypeptide composition of antigen 2 immunoprecipitated in CIE gels, analyzed by SDS-PAGE and immunoblotting after excision from the gel, further revealed that it comprised the same two prominent antigens with molecular masses of 33 and 38 kDa.

Thus it was demonstrated that one antigen of *B. gingivalis* showing erythrocyte-binding capacity, the hemagglutinating adhesin (HA-Ag2), comprises a protein complex composed of at least two associated polypeptides with molecular masses of about 33 and 38 kDa. There seems to be no evidence of a disulfide linkage between these two polypeptides, since their migration in SDS-PAGE is unaffected by the omission of β-mercaptoethanol from the dissociation buffer. The polypeptides at 33 and 38 kDa were also antigenically similar, and their relatedness resulted from sharing at least one epitope. The observation that several bands on immunoblots are derived from the same antigen and bear the same epitope also suggests that this epitope is repetitive rather than conformational. The polypeptides at 33 and 38 kDa were also common to at least three strains of the species *B. gingivalis*, namely, strains ATCC 33277, 381, and W83, further supporting the previous observation (20) that antigen 2 is an antigen common to the species. Data collected in this study are insufficient to unambiguously characterize the relationships of the 33- and 38-kDa subunit proteins to each other and to HA-Ag2. Further biochemical studies, for instance, peptide mapping by limited proteolysis and subsequent analysis by SDS-PAGE, are clearly needed to establish whether the antigenically related bands are distinct proteins differing in mobility due to a distinct chemical composition, whether the smaller and larger polypeptides are related by a precursor-product relationship, or whether they differ in mobility as the result of artifactual proteolysis during preparation.

Our observation of two associated polypeptides with molecular masses of about 33 and 38 kDa is at a slight variance with that of Naito et al. (16), who reported that monoclonal antibody C1.17 reacted with antigens at 40 and 60 kDa. They observed this reaction with blots prepared from a sonic extract, an EDTA cell surface extract identical to ours, and a hemagglutinin-enriched fraction. A close examination of their report indicates that two bands were indeed revealed but between the 49.6 and 37.2 kDa markers. Thus if the lower band is 40 kDa, possibly corresponding to the 38-kDa band of our study, the higher band cannot be 60 kDa. Such discrepancies between the two studies may partly be explained by the use of minigels by Naito et al. to produce

blots and by the use of molecular mass markers from different sources. Further studies are required to establish whether the HA-Ag2 identified in this study is identical to the hemagglutinins described by others (9, 16, 19).

A significant observation was that the erythrocyte-binding activity of HA-Ag2 could not be associated with the fimbrilin described by Yoshimura et al. (26), which is characteristic of the species. First, electron microscopic observation of *B. gingivalis* cells incubated with the gold-TSEM probe revealed that a diffuse material lying at some distance from the outer surface of the bacteria was endowed with adhesive activity. Noteworthy was the observation that no fibrillar material reminiscent of fimbriae appeared to be labeled. Second, it was verified that the fimbria-enriched preparation was devoid of hemagglutinating activity, as already reported by Yoshimura et al. (26). Third, HA-Ag2 could be resolved by SDS-PAGE and immunoblotting as two polypeptides with apparent molecular masses (33 and 38 kDa) distinct from the polypeptide appearing in the 41- to 43-kDa region typical of the fimbrial preparation (Fig. 5). Finally, no ELISA inhibition of the antiserum monospecific for HA-Ag2 was obtained with the fimbrial preparation. This series of observations strongly suggests that HA-Ag2 is not the fimbrilin molecule itself. Alternatively, it may be considered that the hemagglutinin of *B. gingivalis* is some fimbria-associated protein, although data from ELISA inhibition and electron microscopic studies seem not to support this view. Other mannose-resistant nonfimbrial adhesins that cause agglutination of erythrocytes have been described, for instance, in *Vibrio cholerae* (6) or in *Escherichia coli* (10); their presence seemed essential for the pathogenesis of cholera or pyelonephritis. Further studies of the hemagglutinating adhesin described in this report should determine whether this adhesin is involved in promoting subgingival colonization by *B. gingivalis* and thus assess its contribution to periodontal pathogenesis. The potential multifunctionality and immunogenicity in humans of this adhesin are currently under investigation.

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